Pestic. Sci. 1998, **54**, 327–337

High-Throughput Screening as a Tool for Agrochemical Discovery: Automated Synthesis, Compound Input, Assay Design and Process Management†

Stuart M. Ridley,* A. Clare Elliott, Mabel Yeung & David Youle

Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire RG42 6EY, UK

(Received 18 June 1998; accepted 14 August 1998)

Abstract: High-throughput screening (HTS) is becoming increasingly important within the agrochemical industry as a means of sustaining and improving the flow of novel products to the marketplace. Technology changes within agrochemical research have to some extent followed progress in the pharmaceutical industry in adopting in-vitro-based HTS strategies for lead discovery. However, agrochemical invention faces the unique challenge of combining the opportunities made available by HTS technology with the key advantage of being able to work in the laboratory with real, commercial, whole-organism targets. This paper describes approaches taken within Zeneca to harness these opportunities effectively through development of novel assay procedures for agrochemical discovery. Quality and quantity of chemical input to the HTS process is of paramount importance for the successful discovery of leads. Approaches employing the application of automated chemical synthesis to achieve this aim are reviewed. Adoption of a high-throughput screening strategy requires a fundamental change in areas such as process management, data manipulation and data analysis. Some of our experiences to date in managing these issues are described. © 1998 Society of Chemical Industry

Pestic. Sci., **54**, 327–337 (1998)

Key words: agrochemical discovery; automated synthesis; robotics; assay design and management; HTS *in vivo* and *in vitro*

1 AUTOMATED CHEMICAL SYNTHESIS

1.1 Introduction

It is becoming increasingly difficult to discover and take to market new agrochemicals, due largely to increasing environmental and regulatory pressures and the highly competitive nature of the agrochemical market. In an effort to maximise the chances of discovering new active compounds, the emphasis in recent years has been on increasing the number of compounds tested, both on the primary high-throughput screens, and on follow-up screens.

The input to these screens is currently derived from several sources, including the exchange of compounds from other companies and natural product extracts, in addition to the compounds prepared by chemists in-house. In the future it is probable that compounds made internally will have to provide a larger proportion of the total number screened. It is therefore essential that the rate of synthesis of exploratory compounds is increased commensurately to accommodate this.

Combinatorial chemistry has emerged over recent years as a very powerful means of producing large numbers of compounds for screening. Although, in the

* To whom correspondence should be addressed.
E-mail: Stuart.Ridley@AGUK.Zeneca.Com

† One of a collection of papers on various aspects of agrochemicals research contributed by staff and collaborators of Zeneca Agrochemicals UK and Zeneca Ag Products USA. The papers were collected and collated by Dr B. C. Baldwin and Dr D. Tapolczay.

 Pestic. Sci. 0031–613X/98/$17.50. Printed in Great Britain

pharmaceutical sector, combinatorial methods of synthesis are now well established, constraints imposed by the quantities of compounds required for screening have, until the advent of high-throughput screening, precluded the application of combinatorial synthesis in agrochemical research. Combinatorial chemistry, increasingly using solid-phase methods, is now recognised as having an important part to play in the production of compounds for screening, particularly in lead generation and as input to the high-throughput in-vitro screens.

Traditional methods of chemical synthesis are of relatively low throughput. A good skilled experimental chemist might typically produce 100 target compounds for screening per year, which is clearly insufficient, so automation of the chemical synthesis becomes essential. The hope is that by screening large numbers of compounds, there will be a corresponding increase in the number of active areas of chemistry discovered. Automation will also be required to follow up the leads from these areas. The biological results from such chemistry can then be obtained more rapidly, together with assessment and decisions on the potential of a particular lead. The ultimate consequence of effective automated synthesis is a decrease in the time taken to get a product to market. This translates to a significant cost benefit in increased patent lifetime for the product.

1.2 Solution phase robotics

1.2.1 Automated synthesis using Zymark robotics

Until recently, automation at Jealott's Hill has involved solution-phase robotics, mainly for lead optimisation, but also for the production of solution-phase combinatorial libraries.

The use of robotics for the solution-phase synthesis of compounds for screening was pioneered in the UK by Zeneca Pharmaceuticals using Zymark robotics.[1] Since 1995 we in Zeneca Agrochemicals have used and developed similar systems for the synthesis of exploratory agrochemicals. The first of the current two systems is depicted in Fig. 1. The central Zymate™ arm is able to access a number of different workstations, set up to enable the robot to mimic the operations carried out in the laboratory. Thus, in a typical run, the starting materials are weighed off-line into the tubes. A solution of the second reactant is dispensed via syringe into each tube, all manipulations of the tubes being carried out by the robotic arm. The resultant mixture may then be heated and/or stirred for a pre-programmed time before product isolation by standard liquid-liquid extraction techniques.

1.2.2 Capabilities of the Zymark robot

Using this first system, up to 50 reactions may be run in a single batch. The scale typically is millimolar, which affords in the order of 100–500 mg final product, easily

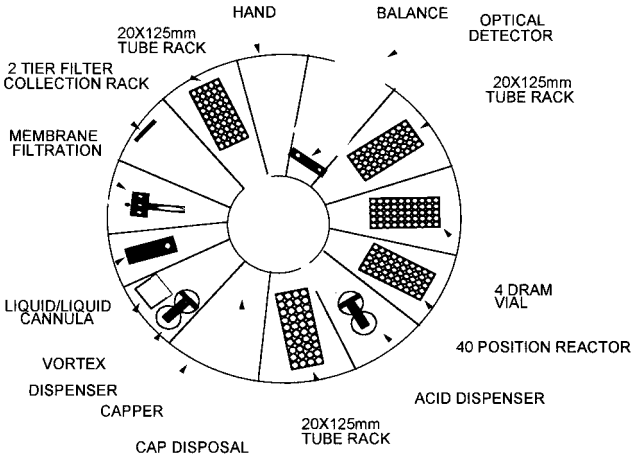

Fig. 1. The Zymark robotic synthesis system used at Jealott's Hill.

sufficient for complete biological screening. The reaction mixtures may be heated up to 150°C. Blanketing of the tubes with argon enables the use of some air-sensitive reagents, such as Grignard reagents, lithium hexamethyldisilazide and potassium *tert*-butoxide.

The second system, recently installed, expands on the first system. In addition to increased capacity, there is now an option to cool to −20°C and to allow the subsampling of all reaction products directly into 96-well microtitre plates. It has been set up on a track rather than a radial base, enabling further workstations to be developed as necessary.

The robot is programmed by linking the individual programmes for each operation in the required order, and by incorporating such data as the number of tubes, heating times and the starting compound weights; using these weights the robot is then able to dispense accurately the required number of equivalents of standard solutions of further reagents. The programming also enables 'scheduling' of the operations, so that they are carried out in parallel rather than sequentially, thus saving time. We have successfully carried out two- and three-step reaction sequences with and without intermediate purification. It is also possible to set up 'matrix' reactions. Rather than reacting a single starting material with each of 50 reactants, six of one type of reactant, for example, are reacted with eight of the second type. This more closely mimics the approach taken in a 'traditional' lead optimisation programme.

Although there are some limits to the range of chemistry that can be carried out on the robot, over the last two years the use of robotics has had a dramatic impact on the number of compounds produced for screening: productivity of the first robot averaged about 2000 reactions run per annum, of which more than 85% gave products suitable for screening. This should increase further with the use of the second robot and greater acceptance by the chemists of the opportunities afforded by automation.

1.2.3 Examples of chemistry carried out on the Zymark robot at Jealott's Hill

- Synthesis of secondary amines from halides and primary amines
- *N*-Alkylation of heterocycles
- Synthesis of carbamates from carbonates
- Synthesis of salts
- Arylation of anions e.g. those formed using lithium hexamethyldisilazide
- Amide formation from acid chlorides formed *in situ*
- Synthesis of α-amino-amides from α-chloro-amides
- Synthesis of mixed anhydrides from acids
- Use of Grignard reagents
- Biotransformations

1.2.4 Supporting systems

Automation of the synthesis of compounds for screening may create bottlenecks in other parts of the synthesis and screening process. It is essential to consider the implications of the increasing numbers of compounds and associated data from the outset and to develop systems to cope with them. The major elements which we have considered are:

- efficient supply and retrieval of starting materials for synthesis;
- electronic data handling and recording of data and procedures;
- automation of analysis;
- automated chromatography; and
- electronic registration of the products into the internal database.

Some of these aspects are considered in more detail below.

1.2.4.1 Electronic data handling and registration. From the outset it was the aim to store as much data as possible electronically, and to devise a procedure for recording and searching which would involve the minimum possible drawing of chemical structures (and certainly no duplication), yet still meet all the necessary legal requirements.

The central repository of the data is an EXCEL spreadsheet into which are entered data relating to the starting materials (directly from an electronic database) and data from the robot relating to the final products. Data collected during GC analyses of the reaction mixtures are also transferred directly to the spreadsheet. An electronic notebook is used by the robotics team to record the experimental procedures, the majority of the information relating to structures having been provided electronically by the customer chemist. This minimises the time taken to draw reaction schemes and structures, and to write up the experiments. The completed spreadsheets and experimental procedure are printed, signed and retained by the customer chemist, a minimum of paperwork being kept within the robotic synthesis team.

Registration of the compounds into the internal database is done directly as a batch from the information recorded in the spreadsheet and this information is transferred electronically to the biologists for screening.

1.2.4.2 Automated chromatography. A large proportion of the chemistry which has been carried out on the Zymark system is concerned with optimisation around a lead in an established area of interest; in such cases, it is important that the final products from the robotic synthesis are sufficiently pure for meaningful structure–activity relationships to be deduced. It is also important in reaction sequences involving several steps that the intermediates produced are pure before submission to the next reaction.

Purification of 50 compounds is potentially highly time-consuming and the development of efficient, automated systems for chromatographic purification of the reaction mixtures has been a priority. The first system used has recently been superseded by a larger arrangement, developed in conjunction with Zymark, in which up to 40 compounds can be purified in a single batch using BondElut chromatography. Up to 10 fractions can be collected from each sample, using gradient solvent elution.[2] Analysis of the fractions is by TLC using an automated system developed in conjunction with Anachem.

1.3 Solid-phase automation

1.3.1 Advantages of solid-phase chemistry

The Zymark robot has been used for the synthesis of solution-phase combinatorial libraries, usually as mixtures; this was seen as the only way to produce large numbers of compounds on a medium-throughput system such as this. Some 18 000 compounds have been produced in this way. However, it is necessary to deconvolute mixtures which prove active, which is time-consuming and does not always result in the identification of single compounds with sufficient activity. Solid-phase chemistry offers a number of advantages over solution-phase chemistry for the efficient production of large numbers of compounds. An excess of reagent can be used in the reaction to drive it to completion, unreacted reagent being removed by thorough washing of the resin after reaction, so that there is less need for purification of the final products. Solid-phase chemistry is highly amenable to automation and a number of instruments are now available for this purpose.

1.3.2 Automation of solid-phase chemistry

Multiple parallel synthesis (MPS) systems typically carry out hundreds of reactions per week of the same order as the Zymark. Split-and-mix technology[3] offers the potential to produce many more compounds rapidly: thus a 10 × 10 × 10 library (1000 compounds)

can be prepared by split-and-mix using only 30 different reactions. Since each bead carries a single compound, screening the compound from a single bead removes the need to deconvolute the mixture after screening.

The major problems in using split-and-mix for the production of exploratory agrochemicals are producing single beads large enough to carry sufficient compound for screening, and having some method for identification of the compound on each bead. Current advances in the automation of solid-phase chemistry are focusing on instruments for multiple parallel synthesis. Automation in support of split-and-mix is less advanced, for example, in technology for bead arraying, resin mixing and splitting, and most importantly for the tagging and tracking of each bead through the synthesis. These are likely to be areas of rapid development in the near future.

2 HIGH-THROUGHPUT SCREENING *IN VIVO*

Currently, high-throughput assays are run *in vitro* and *in vivo* in parallel at 100 000 compounds per assay per year. The defining difference between the two types of assay is the target: in-vivo assays use whole organisms, while in-vitro assays use enzymes, receptors, cells or organelles.

2.1 From assay development to implementation

Conventional in-vivo assays, which screen 5000–10 000 compounds per assay per year are generally laborious, time-consuming, resource-demanding and not suitable for handling large numbers of compounds. Although there is a rapidly evolving spectrum of in-vitro high-throughput screens, there are limited publications on in-vivo high-throughput assays. In modifying conventional screens or developing novel assays to fit a high-throughput format, we have had to identify our own critical success factors and rules to guide the process.

2.1.1 Critical success factors

Miniaturisation and automation have been identified as the key factors for achieving a high-throughput screening rate without increasing resource or laboratory space. The need for only small amounts of compound makes it more possible to achieve an input supply of high quality and diversity.

2.1.1.1 Miniaturisation. Miniaturisation allows us to use less material and physical space. In-vivo assays that once required 20–30-ml containers are now done in one-hundredths of this volume. In addition to lower cost, being able to use less biological material is particularly important for in-vivo screens; bulk production of whole plants or insects, followed by freezing for storage before use, is not possible. Timeliness is important. The pressure on the culturing service to provide the plant or insect at exactly the right moment and at exactly the right life stage is immense. The use of less biological material helps to alleviate that pressure.

2.1.1.2 Automation. Well-planned automation saves both labour and time. Automation includes simple hand-held equipment, modular equipment requiring operator initiation and intervention, and full automation which can carry out the screening operation unattended whilst providing the option of running assays around the clock.

Screens are now amenable to technology based on the microtitre plate. Automation can improve the assay quality by standardising routine operations (e.g. liquid-handling robots capable of aspirating and/or dispensing liquids), reducing person-to-person variation and subjective judgement (e.g. the use of common readers and detection devices for assessment) and in reproducibility (e.g. electronic scheduling to maintain precise incubation timing).

2.1.1.3 Low compound weight requirement. New methods have emerged for generating compound libraries rapidly as a source of input for lead generation and lead optimisation programmes. The growing trend for synthesising and submitting smaller quantities of compounds ultimately limits their availability for more extensive screening. Lower compound requirement (mg, or even μg) increases the flexibility for negotiating acquisitions (e.g. exchange of compound collections with other companies or research institutes), and also allows us to tap new sources of chemicals for diversity. With such small amounts of compound available, more efficient data management, storage and distribution are required.

2.1.2 Rules

The agrochemical discovery process is a multidisciplinary operation for detecting fungicidal, insecticidal and herbicidal activity. The spectrum of target organisms and their biology is very diverse, and flexibility in running a streamlined and efficient operation is needed. Rules for the design of high throughput in-vivo screens must therefore be established.

Potential. A basic experimental design should be amenable to scale-up and automation.

Set-up. An optimised experimental design should be simple to perform and analyse, and many compounds should be tested during each run.

Resource requirement. Each compound should have a minimum requirement for labour, space, time, cost, material and equipment.

Format. A standardised format should be compatible with microtitre plates to make work faster and more productive. For example, the same chemicals are supplied in 96-well matrix to all assays. This means that

multiple daughter plates are made from one mother plate by simple copying, without any time-consuming or complicated re-formatting.

Controls and standards. Reagent controls and chemical standards should be included for quality assurance. An assay should detect both common agrochemical standards and weak leads at a required level of potency for an interesting lead. Controls and standards allow us to check the chemical preparation procedure and compare results over several months, and also to check for systematic effects and variability.

Compound identification. Each compound should have a unique identifier, allowing tracking of progress. One primary role of the microtitre plate is the assignment of identity to any compound within it.

Data summary. Powerful technology is available for assay assessment. The readout may be radioactivity, optical density, visible coloration, movement, plant damage, percentage reduction or straight numbers; but all readouts should be summarised into three levels of chemical activity: inactive, low activity or high activity. This simplifies manual assessment and data capture for the experimentalists while facilitating a cross-disciplinary approach for interpreting and understanding the results by general users.

Data capture. All assays should use one centralised chemical treatment list and the same data capture programme. Software programmes should be written in-house.

Hit rate. It is difficult to predict the hit rate since it depends on the chemical input and biology. Nevertheless, assay design should reflect practical considerations such as the capacity of the follow-up assays to handle the volume of active leads.

2.1.3 Assay development

The success of in-vivo screening depends on gaining the maximum value from every assay. This can only be achieved by having (a) an appropriate selection of targets; (b) a diverse set of compounds available for screening; (c) the best tools to design assays compatible with the demands; (d) stringent validation to ensure the quality of the resulting data; (e) an efficient data management system to manage the very high volume of information generated by the new technology; (f) timely access to the information and (g) the data presented in the best way to allow decisions to be made quickly and to avoid missing any new opportunities.

2.1.3.1 Target selection. Choosing a screen target is important because it addresses the question of whether to use the real pest as target, or to use an indicator species as a model for the real target. Indicators are chosen only when they have obvious advantages such as ease of mass culturing, good chemical detection that correlates with real pest targets, and amenability to high-throughput screens. For pharmaceutical drug discovery, the main target organism is man and there is a concerted effort by the human genome project to complete the sequencing by 2002. However for agrochemicals, the target organisms are much more diverse. The model organisms are either fully sequenced (e.g. yeast) or partly sequenced (e.g. *Arabidopsis*, *Drosophila*, *Caenorhabditis*), but the genome information for most target pests is generally not available.

2.1.3.2 Basic experimental design. To start a new screen, the optimum conditions must first be established. Experimentalists choose a number of factors (generally based on literature review, in-house expertise and past experimental results) that they believe may have an effect on the response. All factors are varied simultaneously in a systematic way, including the extremes of the operating range. This will allow the significance of any one specific factor (or interaction between factors) to be measured and optimised. Scale-up and automation of the screens must avoid changes in the basic assay properties. For example, changes involving the order of infestation or compound application can have a major impact on the relevance of results.

2.1.4 Assay validation

A stringent procedure is needed to indicate whether an assay is of acceptable quality, particularly if each compound is tested without replication. The three criteria are:

Robustness. Experiments should be designed to test the endurance of the procedures to ensure that variation of response from one week to the next is minimal, and that standard test results are consistent and sustainable over time.

Reliability. Reliable detection of chemical standards and signals for weak leads requires understanding of the biology of the target, the host (where appropriate) and the chemistry to highlight any peculiarities.

Reproducibility. Testing control or standard compounds every time the screen is run provides a useful estimate of variability from standard deviations.

2.1.5 Assay implementation

We need to ensure that screen assays run within the required specifications sets. Different criteria of timescale, resource, support and the type of expertise involved will apply. It is important to be aware of this to ensure a smooth transition from a small-scale development phase to a phase of large routine screening. Examples of Jealott's Hill of whole organism high-throughput screens are given below for the various disciplines:

2.1.5.1 Fungal control. Six high-throughput bioassays are currently in place, employing pathogens representative of all four of the major taxonomic subdivisions of

the eumycotic fungi. Two general methods of bioassay are used:

(i) high-throughput microtitre plate-based bioassays that test chemicals for inhibition of fungal growth using calibrated preparations of inoculum suspended in a defined minimal medium;
(ii) high-throughput seedling-based assays to find chemicals that will prevent the successful colonisation of a host by a range of obligate biotrophic plant pathogenic fungi. These assays detect activity attributable to uptake of the applied compound into plant and fungal cells following foliar deposition and/or drench treatment of the plant roots.

Similar methods are then used to characterise the antimicrobial spectrum and potency of active compounds. These provide dose-response information across a spectrum of 13 target species, using less than 2 mg of chemical in total.

2.1.5.2 Weed control. High-throughput in-vivo assays have been developed involving a range of weed species contained within deep and shallow well plates. Chemicals are transferred to test plates which are filled with agarose agar (8 g litre^{-1}) to the level required for quality plant growth. The seed species are applied manually using a matrix grid which varies according to the size of the seed. The assay plates are sealed and contained under controlled conditions in a growth chamber for seven days, after which a semi-automated assessment is carried out on chemical damage and plant symptomology.

2.1.5.3 Insect control. Three high-throughput, *in-vivo* assays are run that include two target pests and one indicator species. The assays are carried out in 96-well plates. The test plates are prepared by dispensing a standard volume of insect diet medium into each well and then infesting with the individual insect species. For each assay, chemical plates are prepared as single rates for primary screening, and as a dose-response for follow-up and potency screening. The chemicals are transferred by an automated liquid handling machine from a daughter plate onto the test plate containing the insects. The test plates are sealed to prevent insect escape and kept under controlled conditions. Four or six days after chemical application, an automated assessment is carried out on movement and diet consumption.

2.2 Impact of high-throughput screening on the discovery process

The modern discovery process can be compared to a funnel into which hundreds of thousands of compounds are poured each year in the hope of discovery new leads. The process involves a screening cascade of several levels where compounds are tested, and the most promising leads progressed to the next level, which ultimately might lead to the market. Making the right decisions requires timely access to all the appropriate information in a simple format.

2.2.1 Management of compounds and data

To ensure that the discovery process flows smoothly, advanced compound-handling and data-management techniques must be in place to screen and process the huge number of results generated by high-throughput screening.

2.2.1.1 Compound input. High-throughput screening in microtitre plates has made possible the screening of huge numbers of samples, and the concept of screening millions of chemicals has been introduced through combinatorial chemistry. It has also allowed a greater diversity of sample input within a given time compared to conventional low-throughput screening. The input includes compounds generated by combinatorial chemistry, mixtures and natural products, as well as individual discrete samples. Compounds may be contained individually in vials or pre-loaded on 96-well plates from commercial suppliers, collaborators, exchange or in-house synthesis.

2.2.1.2 Automated sample-handling system. A modular automated sample-handling system in a Dispensary unit eliminates the high labour burden and sample-handling errors which are inevitable with a purely manual system. Bar code labelling minimises errors in the tracking of samples for use.

2.2.1.3 Tracking. The entry point into the discovery screening process is through the microtitre plate issued by the Dispensary. Data management systems need to track the identity, source, history, ownership and the activities of compounds in each individual well, because plates are distributed to different locations for different assays. Each plate has a specific identifier, and each well has a unique set of data and tracking information. Tracking is achieved by connecting the automation for sample and plate-handling directly to our own compound and inventory databases to locate samples for experiments. It allows the Dispensary to create mother, daughter and assay plates, and to monitor closely key information and the assays performed.

2.2.1.4 Data package. The individual assays are linked together through a central screening data server, and an in-house data package has been written which contains all data on an individual sample. This has been integrated into our existing information system, and allows retrieval of chemical structures and other information such as physical properties, cross-reference to publications, screen history, and cluster information, all of which can be combined with the biological and biochemical assay results. The data package allows the

data users to view simultaneously and in a timely manner all the information for a single compound across all assays from different laboratories.

2.2.1.5 Data analysis. Raw data are transformed into summary level data, and this is followed by extraction of patterns and information. Testing of combinatorial libraries requires special consideration, such as multivariate visualisation as a tool for rapid identification of patterns in the data. The challenge is the huge volume of data available. An automated, rule-based software (written in-house) improves the consistency of data analysis done by different people by applying a standardised set of criteria. It reduces repetitive steps (quickly clustering the data into groups for curve fitting), and identifies data which require more intensive manual analysis.

2.3 Screen correlation

2.3.1 Screening in vitro *and* in vivo

In-vitro assays are valuable for understanding the intrinsic activity on well-validated targets. They are also more amenable to further miniaturisation. In-vivo assays, on the other hand, cannot be miniaturised to the same degree but have the advantage that information is on whole organisms and thus will cover all potential modes of action, including the possibility of host interaction.

2.3.2 High throughput and glasshouse screening

A better understanding of the correlation of all level of screens in the cascade is necessary because, ultimately, high-throughput leads will need to be progressed to glasshouse screens which predict activity in the field. The screening cascade fails if the leads are active only on the initial high-throughput tests but inactive on glasshouse screens, or if the ranking of activity for a group of compounds differs greatly at the various levels of screening. Re-examination of the glasshouse screens is costly in terms of resource, whereas less resource is needed for large-scale studies of high-throughput assays.

3 HIGH-THROUGHPUT SCREENING *IN VITRO*

An *in-vitro* screening rate of hundreds of thousands of compounds a year against a number of carefully selected targets gives us a good opportunity to discover new chemical types of interest through the use of three different types of assay: colorimetric, radioligand binding, and cell-based whole-pathway reporter screens. Examples of some of the assays we have run are given below.

3.1 Colorimetric assays

3.1.1 Direct reading of substrate or product

Some enzyme assays may be recorded directly through the disappearance of a substrate or the appearance of a product, and these are particularly amenable to high-throughput screening. For example: (a) electron transport in mitochondrial respiration can be measured by recording the oxidation of NADH at 340 nm; (b) in an assay for δ-aminolaevulinic acid dehydratase, an early step in the biosynthesis of tetrapyrroles, the product, porphobilinogen, can be measured directly at 550 nm. Such assays require no further addition.

3.1.2 Visualisation of a phosphate product

Most target-site reactions for high-throughput screening need additions that will convert the product, or react with it, and generate an entity that can readily be measured spectroscopically. One widely used procedure relies on the production and measurement of inorganic phosphate (P_i). We have used the technique involving Malachite Green which gives a colorimetric end-point at 630 nm.[4] This is highly sensitive, so all ingredients must be entirely phosphate-free and the enzyme preparations must also be free of non-specific phosphatases.

The following are some examples of where we have used a modification of this.

(a) Acetyl-CoA carboxylase, the fist enzyme in the biosynthesis of fatty acids and the site of action of the aryloxphenoxypropionate and cyclohexanedione herbicides:[5]

$$\text{ATP} + \text{CO}_2 + \text{biotin carboxyl carrier protein (BCCP)} \rightarrow \text{CO}_2\text{-BCCP} + \text{ADP} + \text{P}_i$$

$$\text{CO}_2\text{-BCCP} + \text{acetyl-CoA} \rightarrow \text{malonyl-CoA} + \text{BCCP}$$

(b) Glutamine synthetase:

$$\text{L-Glutamate} + \text{ATP} + \text{NH}_3 \rightarrow \text{Glutamine} + \text{ADP} + \text{P}_i + \text{H}_2\text{O}$$

(c) Farnesyl pyrophosphate synthase (FPPS):

$$\text{IPP} + \text{GPP} \rightarrow \text{FPP} + \text{PP}_i$$

$$\text{PP}_i \rightarrow 2\text{P}_i$$

Where pyrophosphate (PPi) is generated we have also determined the phosphate generated. In the assay for FPPS an inorganic pyrophosphatase is included in the assay mixture for indirect generation of phosphate (Hughes and Ridley, unpublished). Isopentenyl pyrophosphate (IPP) and geranyl pyrophosphate (GPP) produce farnesyl pyrophosphate (FPP) and inorganic pyrophosphate. The latter is converted to inorganic phosphate.

3.1.3 Visualisation in other assays

A number of other screens involving colorimetric visualisation of product have been run. For example:

(a) Glycolate oxidase in the photorespiration cycle:

$$\text{Glycolate} + O_2 \rightarrow \text{Glyoxylate} + H_2O_2$$

The hydrogen peroxide generated is detected using 4-aminoantipyrene, horseradish peroxidase and phenol, which are included in the incubation mixture and form a red chromagen absorbing at 505 nm.[6]

(b) Imidazoleglycerol phosphate dehydratase in the histidine pathway, which converts imidazoleglycerol phosphate (IGP) into imidazoleacetol phosphate (IAP), has been measured in a coupled assay using glutamate dehydrogenase so that the oxidation of NADH is finally recorded.[7]

$$\text{IGP} \rightarrow \text{IAP [IGP dehydratase]}$$

$$\text{IAP} + \text{Glutamate} \rightarrow \text{Histidinol-P} + \alpha\text{-ketoglutarate } (\alpha\text{-KG}) \text{ [IAP transaminase]}$$

$$\alpha\text{-KG} + \text{NADH} \rightarrow \text{Glutamate} + \text{NAD [glutamate dehydrogenase]}$$

3.1.4 Modification of substrate and product

One assay that we have run has used modification of the substrate in addition to recording the generation of phosphate from pyrophosphate. This is isoleucyl-tRNA synthetase, the site of action of the natural product antibiotic pseudomonic acid A (produced by *Pseudomonas fluorescens* Mig.), and hence an inhibitor of protein synthesis. Hydrolysis yields monic acid A, various derivatives of which display useful levels of herbicidal activity (unpublished).

The assay is usually carried out using radiolabelled isoleucine, but in order to transform it into a high-throughput screen, we have taken advantage of the fact that the isoleucyl enzyme will also recognise valine as a substrate.[8]

$$\text{Enzyme} + \text{ATP} + \text{val} \rightarrow \text{Enzyme : val-AMP} + \text{PPi}$$

$$\text{Enzyme : val-AMP} \rightarrow \text{Enzyme} + \text{val} + \text{AMP}$$

The enzyme catalyses the hydrolysis of the non-cognate aminoacyladenylate bound to the enzyme, but there is no transfer to the ^{isoleu}tRNA, so only catalytic amounts of tRNA are required; the starting enzyme and valine are regenerated while generating pyrophosphate. The inclusion of an inorganic pyrophosphatase in the reaction mixture allows phosphate to be generated and determined as a measure of the overall reaction. Compounds showing inhibition activity are then checked against the enzyme using the labelled true substrate, [^{3}H]isoleucine.

3.2 Radioligand assays

Certain screens cannot readily use colorimetric methods for measurement, and so radioactive substrates have to be used. This has been particularly important in screens involving insect nerve receptors where the binding of an appropriate radio-labelled nerve toxin may be displaced by a potential novel inhibitor. Radio-labelled ligands such as [^{3}H]bungarotoxin (a nicotinic acetylcholine receptor inhibitor) and [^{3}H]quinuclidinyl benzilate (a muscarinic acetylcholine receptor inhibitor) are incubated in 96-well microtitre plates together with isolated nerve receptors and test compounds. The contents of the plates are transferred to glass-fibre mats and washed using a cell harvester; the radioactivity on the mats is then counted. An active compound will be detected by the reduction or absence of radioactivity.

3.3 Whole-cell assays

An example of a whole-cell assay is a sterol reporter gene assay which was designed to identify potential modulators of fungal sterol biosynthesis.[9] It makes use of the fact that an end product of the sterol pathway (ergosterol) has a feed-back control on the promoter gene for the first enzyme, acetoacetyl-CoA thiolase, in the common pathway for isoprenoid biosynthesis.

The screen uses whole yeast cells (*Saccharomyes cerevisiae* Mayer ex Hansen) which have been genetically modified so that the promoter gene for acetoacetyl-CoA thiolase is fused to a reporter gene encoding β-galactosidase (β-gal); this promotor will also activate the β-gal gene.

The β-gal gene is promoted if no sterol binds to the receptor-binding protein, in which case β-galactosidase will be produced. This is true if one of the enzymes in the sterol pathway is inhibited. The β-gal enzyme is then presented with a substrate (chlorophenol red-β-galactopyranoside or CPRG) from which chlorophenol red is enzymatically liberated. So inhibition of the sterol pathway will be signalled by a red colour in the microtitre plate.

With no sterol inhibition there will be no β-gal activation, and only an orange/yellow colour will be obtained. Inhibitors of protein synthesis that block the transcription or translation of the β-galactosidase biosynthesis when the β-gal gene is activated will also give a yellow colour, so only the truly positive inhibitors of the sterol pathway will be detected.

3.4 Support for a high-throughput in-vitro screening operation

The running of a high-throughput in-vitro screening operation requires dedicated support in terms of a regular supply of materials (enzymes, receptors, substrates) to ensure the continuity of the screens. This

also involves an awareness of new technology for enhancing the screening operation. Where possible, target enzymes are over-expressed in a micro-organism or single cell to allow large-scale (10-litre) fermenter growth. Development of purification protocols for each target is then researched. Such a process requires the careful integration of joint forces from biotechnology, microbiology and biochemistry.

There are some target sites that are not easy to over-express genetically because they involve several proteins. This is true of certain nerve receptors needed to screen for insect control. In this event the original material must be extracted directly from the insect as described in outline below:

3.4.1 Fly head and nerve receptor production

Flies (*Lucilia sp.*) are hatched from pupae over about five days at 25°C. They are then frozen and the heads separated from the bodies. The heads are homogenised and the nerve receptors isolated by differential centrifugation.

3.4.2 Translation of assays to high-throughput screens

Assays developed in a test tube cannot necessarily be converted directly into screens in microtitre plates. Research is needed to ensure that each assay is adaptable to automation, and is sufficiently robust to enable the screening of at least 100 000 compounds. The number of additions in the assay must be kept to a minimum, and background readings kept low. A reduction in volume, whether in 96- or 384-well plates, can also introduce problems of adequate mixing. It is important to have robust data capture and management facilities in place.

3.5 Progression, and the discovery of new targets

3.5.1 Potency testing

Compounds giving greater than 80% inhibition at the chosen screening dose on an in-vitro assay are subjected to a dose-response test to establish an IC_{50} value. Where a compound also registers biological activity, the in-vitro potency can be related to the in-vivo dose response, since there is always the possibility that the biological effect may be caused primarily by action at a different site.

3.5.2 Mode-of-action

Potent inhibitors of a biological screen that do not record a hit in any of the *in-vitro* assays may have a novel mode of action. Such compounds, if deemed particularly interesting in terms of chemical structure and biological efficacy, are passed through a number of individual assays appropriate to the particular discipline, and these include broad (whole pathway) assays as well as specific tests. If these are negative, the compound may ultimately enter a research programme to establish its action, and this could involve genomic microarray to highlight changes in gene expression.

4 THE FUTURE VISION

4.1 Future directions in the automation of synthetic chemistry in agrochemical research

Combinatorial technology offers the opportunity to produce large numbers of compounds as input for high-throughput screens. However, combinatorial chemistry has been used in agrochemical research only during the past few years and it is too early to be sure that this approach will indeed deliver more active compounds into the process. Increasingly, emphasis is being put on the importance of the design of the combinatorial library; computational tools are being developed which take account of such factors as the geometry of the target site, or ensure that the final products have physical properties known to be favourable for activity. Such knowledge should allow chemists to maximise the potential of the combinatorial approach.

Both split-and-mix and multiple parallel synthesis approaches are being pursued in agrochemical research, each having its advantages and disadvantages. The development of effective automation will be one factor determining the future approach to combinatorial chemistry. In particular, the successful implementation of the split-and-mix approach will depend on the development of rapid, automated and cost-effective methods for identifying the compound on each bead and sorting them. Recent advances in this area include the development of radio-frequency encoded micro-reactors by IRORI;[10] a system has recently been introduced which can automatically sort these after split-and-mix synthesis.[11] Laser optical encoding is another recent approach to efficient non-invasive tagging.[12] The development and increasing automation of the techniques are likely to increase the potential applicability of the split-and-mix approach in the future.

The development of screens which can operated on smaller amounts of chemical will also have an impact on the choice of approach and associated automation. A current problem with screening a compound on a single bead from split-and-mix is one of capacity; it is difficult to develop beads capable of holding sufficient compound for screening and yet robust enough to cope with the reaction conditions during synthesis.

4.2 Lead optimisation

It is hoped that the greater number of compounds entering high-throughput screens will result in more active leads to explore. Using current methods of chemical synthesis, not all the anticipated lead areas can be followed up, so the development of further information to effect high-throughput synthesis is essential.

Systems such as the Zymark robot have proved highly productive for the synthesis of large numbers (50–100) of compounds, usually by a specialist team. There is an increasing need for automated systems, for use by the bench chemist, to produce smaller series of compounds (10–50). Instruments which are user-friendly and able to carry out a greater range of chemistry are now marketed or under development by a number of companies.

4.3 Future in-vivo screening strategy

4.3.1 Broadening the spectrum

We have now shown that high-throughput screening *in vivo* can be achieved. The next step is to develop more assays for a larger number of targets. It is important to prioritise the targets by considering their importance to the agrochemical market.

4.3.2 Automation

Off-the-shelf microtitre plate technology is adequate for the early start-up phase. However, bearing in mind the potential for increase in throughput and number of targets and assays, there is an increasing need to:

(a) identify and prioritise the processes that can benefit from automation;
(b) identify automation that can be shared across similar steps in different assays;
(c) improve the equipment by simple modification, e.g. incorporation of automated stacking systems for moving batches of plates;
(d) develop custom-built equipment for certain processes, e.g. for seed dispensing; and
(e) stimulate the use of professional integrators whose business is the creation and assembly of systems rather than the manufacturing of equipment.

4.4 Divergence of in-vivo and in-vitro screening

At present, it is usual to screen compounds through in-vitro and in-vivo targets at the same rate. However, in-vitro screening techniques are undergoing a revolution in miniaturisation. The standard 96-well microtitre plate will gradually be replaced by the 384- or 1536-well plate. The rate of compound screening will increase, and the amount of materials, including enzyme targets and compounds, will become smaller, thus maintaining a cost-efficient screening strategy. Already there are commercial screening packages on offer that use 'nanoplates' having 3456 wells in each plate, allowing the screening of millions of compounds a year.

All of this would mean a gradual divergence of in-vitro from in-vivo screens in the agrochemical industry. In-vivo screening may encounter difficulties in miniaturising beyond the 96-well format, and screening rates are likely to be constrained by this fact. In-vitro screens, on the other hand, may embrace the new microfluidics and 'nanoplate' technologies and move into ultra-high-throughput screening. For the agrochemical business, the effects of an inhibitor on the biological material are still of paramount importance, but the ability to screen individual carefully chosen enzyme or receptor targets at very high rates may alter screening strategies. Compounds inhibiting both an in-vitro target and the associated biological material are clearly the most accessible to chemical exploitation, but a compound inhibiting a target in the nanomole or even picomole range, without also showing biological inhibition, is clearly a candidate for further study and structural searching.

Further down the screening cascade there are supporting tests such as those for potency (IC_{50} values), mode-of-action and physical properties which are also amenable to miniaturisation, and new technology in micro-engineering is likely to assist the invention process here.

Screening strategies should not be driven simply by the opportunities offered by new technologies, but rather, advantage taken of them to suit the screening strategy. In other words, consideration must be given to what is possible versus what is desirable. Furthermore, screening a million compounds without consideration of 'quality of input' is not likely to be as productive as screening a smaller number of compounds where chemical quality and diversity have been taken into account, and where more thought can be put into mechanism-based design. In spite of these cautions, the agrochemical business will assuredly be pursuing higher rates of both in-vitro and in-vivo screening over the coming years to improve the rate of discovery.

REFERENCES

1. Main, B. G. & Rudge, D. A., Automated chemical synthesis. In *International Symposium on Laboratory Automation and Robotics*, ed. J. R. Strimaitis & G. L. Hawk. Zymark Corp., Hopkinton, MA, 01748-1668 USA, 1994 pp. 425–34.
2. Walter, G. C., Elliott, C. & Higgins, K. F., Internet http://www.islar.com/ABSTR97/Walter.htm.
3. Furka, A., Sebestyen, F., Asgedom, M. & Dibo, G., General method for rapid synthesis of multicomponent peptide mixtures. *Int. J. Pept. Protein Res.*, **37** (1991) 487–93.
4. Hess, H. H. & Durr, J. E., Assay of inorganic and organic phosphorus in the 0·1–5 nanomole range. *Anal. Biochem.*, **63** (1975) 607–13.
5. Howard, J. A. & Ridley, S. M., Acetyl-CoA carboxylase: a rapid novel assay procedure used in conjunction with the preparation of enzyme from maize leaves. *FEBS Lett.*, **261** (1990) 261–4.
6. Turner, J. C. & Hall, N. P., Identification of genetically induced lesions and the sites of action of inhibitors affecting photorespiration by simple tests on leaf discs. *J. Exp. Bot.*, **39** (1988) 345–51.
7. Hawkes, T. R., Cox, J. M., Barnes, N. J., Beautement, L. S., Edwards, L. S., Kipps, M. R., Langford, M. P., Lewis, T., Ridley, S. M. & Thomas, P. G., Imidazole

glycerol phosphate dehydratase: a herbicide target. *Proc. Brighton Crop Prot. Conf—Weeds* (1993) 739–744.
8. Fersht, A. R., Editing mechanisms in protein synthesis. Rejection of valine by isoleucyl-tRNA synthetase. *Biochemistry*, **16** (1977) 1025–30.
9. Dixon, G., Scanlon, D., Casper, S. & Broad, P., A receptor gene assay for fungal sterol biosynthesis inhibitors. *J. Steroid Biochem. Mol. Biol.*, **62** (1997) 165–71.
10. Nicolaou, K. C., Xiao, X.-Y., Parandoosh, Z., Senyei, A. & Nova, M. P., Radiofrequency encoded combinatorial chemistry. *Angew. Chem., Int. Edn Engl.*, **34** (1995) 2289–91.
11. Internet http://www.irori.com
12. Xiao, X.-Y., Zhao, C., Potash, H. & Nova, M. P., Combinatorial chemistry with laser optical encoding. *Angew. Chem., Int. Edn Engl.*, **36** (1997) 780–2.